Coenzyme M binds to a [4Fe–4S] cluster in the active site of heterodisulfide reductase as deduced from EPR studies with the [^{33}S]coenzyme M-treated enzyme

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Abstract Heterodisulfide reductase (Hdr) from methanogenic Archaea catalyzes the reversible reduction of the heterodisulfide (CoM-S–S-CoB) of the methanogenic thiol coenzymes, coenzyme M (CoM-SH) and coenzyme B (CoB-SH). Upon reaction of the oxidized enzyme with CoM-SH a unique paramagnetic species is formed, which has been shown to be due to a novel type of [4Fe–4S]$^{3+}$ cluster. In this work, it was addressed whether CoM-SH is directly attached to this [4Fe–4S] cluster using CoM-^{33}SH as substrate and purified Hdr from *Methanothermobacter marburgensis* and *Methanosarcina barkeri*. With both enzymes treatment with CoM-^{33}SH in the presence of duroquinone as an oxidant resulted in a significant broadening of the electron paramagnetic resonance spectrum as compared to CoM-SH as substrate. The signal broadening resulted from an unresolved anisotropic hyperfine coupling between the ^{33}S nucleus and the paramagnetic center. The results provide compelling evidence for a direct binding of CoM-SH to the [4Fe–4S] cluster in the active site of the enzyme.

Key words: Heterodisulfide reductase; Iron–sulfur protein; Electron paramagnetic resonance spectroscopy; *Methanothermobacter marburgensis*; *Methanosarcina barkeri*

1. Introduction

Heterodisulfide reductase (Hdr) is a unique disulfide reductase, which has a key function in the energy metabolism of methanogenic Archaea. The enzyme catalyzes the reversible reduction of the mixed disulfide (CoM-S–S-CoB) of the two methanogenic thiol coenzymes, called coenzyme M (CoM-SH) and coenzyme B (CoB-SH). This disulfide is generated in the final step of methanogenesis [1]. Two types of Hdr from phylogenetically distantly related methanogens have been identified and characterized. Neither type of enzyme belongs to the family of pyridine nucleotide disulfide oxidoreductases [2].

Hdr from *Methanothermobacter marburgensis* is an iron–sulfur flavoprotein composed of the subunits HdrA, HdrB, and HdrC. The enzyme has been purified from the soluble fraction and none of its subunits are predicted to form transmembrane helices. From sequence data, it has been deduced that HdrA contains an FAD-binding motif and four binding motifs for [4Fe–4S] clusters. HdrC contains two additional binding motifs for [4Fe–4S] clusters [3,4].

Hdr in the two closely related *Methanosarcina* species *M. barkeri* and *M. thermophila* is tightly membrane-bound [5–7]. The enzyme is composed of two subunits, a membrane-bound *b*-type cytochrome (HdrE) and a hydrophilic subunit (HdrD) containing two binding motifs for [4Fe–4S] clusters. Subunit HdrD of the *M. barkeri* enzyme is a homologue of a hypothetical fusion protein of the *M. marburgensis* HdrCB subunits [6]. A homologue of the *M. marburgensis* HdrA subunit is lacking in Hdr from *Methanosarcina* species. From these data it has been concluded that the conserved subunits HdrD and HdrCB must harbor the catalytic site for the reduction of the disulfide substrate [6]. HdrB harbors two copies of the unique $CX_{31-32}CCX_{33-38}CXXC$ sequence motif. This sequence motif is also conserved in the C-terminal part of subunit HdrD of *M. barkeri* Hdr. Some of these cysteine residues have been proposed to ligate an additional iron–sulfur cluster in the active site of the enzyme [6]. Recently we presented evidence for the existence of a novel type of iron–sulfur cluster, present in the active site that is directly involved in mediating heterodisulfide reduction [8,9]. A detailed spectroscopic characterization of the enzyme from *M. marburgensis* and *M. barkeri* was performed. Electron paramagnetic resonance (EPR) signals of potential relevance to the catalytic cycle were observed on reaction of oxidized Hdr with either CoM-SH or CoB-SH, the co-substrates for the oxidative reaction [8]. In the presence of CoM-SH, a novel $S = 1/2$ resonance was observed at temperatures below 50 K, with principal g values of 2.013, 1.991 and 1.938 for Hdr from *M. marburgensis* and $g_{xyz} = 2.011$, 1.993, 1.944 for the *M. barkeri* enzyme. This paramagnetic species has been designated oxidized Hdr incubated with CoM-SH (CoM-Hdr). The resonance is lost on reduction ($E_m = -185$ mV versus NHE at pH 7.6) and on reaction with CoB-SH. Hence, it was attributed to the product of the oxidative half-reaction that occurs in the absence of CoB-SH, in which case it is likely to correspond to a trapped intermediate in the catalytic cycle. A spe-

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Abbreviations: Hdr, heterodisulfide reductase; CoM-SH, coenzyme M or 2-mercaptoethane sulfonate; CoB-SH, coenzyme B or 7-mercaptoheptanoylthreonine phosphate; CoM-S–S-CoB, heterodisulfide of CoM-SH and CoB-SH; CoM-SeH, 2-selanyl-ethane sulfonate; CoM-Hdr, oxidized Hdr incubated with CoM-SH; CoB-Hdr, oxidized Hdr incubated with CoB-SH; FTR, ferredoxin:thioredoxin reductase; NEM-FTR, *N*-ethylmaleimide-modified FTR

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cies with similar g values, $g = 2.018$, 1.996 and 1.954 (Hdr from *M. marburgensis*), and relaxation properties was observed when oxidized Hdr was treated with CoB-SH, designated oxidized Hdr incubated with CoB-SH (CoB-Hdr). However, redox titrations revealed a significantly higher midpoint potential ($E_m = -30$ mV versus NHE at pH 7.6) than the CoM-SH-generated species and argue against a role as an intermediate in the Hdr catalytic cycle.

The CoM-SH-induced EPR signal in *M. marburgensis* Hdr showed ^{57}Fe broadening, indicating it corresponds to a paramagnetic Fe–S center rather than an organic radical [8].

To further characterize this Fe–S cluster, variable-temperature magnetic circular dichroism (VTMCD) spectroscopy studies of CoM-Hdr were performed which showed the presence of a novel type of [4Fe–4S]$^{3+}$ cluster at the active site of Hdr [9]. The VTMCD spectrum of CoM-Hdr is distinct from those associated with the most common types of oxidized paramagnetic Fe–S clusters, i.e. cubane and linear [3Fe–4S]$^{+}$ clusters and HiPIP-type [4Fe–4S]$^{3+}$ clusters [10]. However, the VTMCD spectrum of CoM-Hdr shows correspondence to that observed for the novel type of [4Fe–4S]$^{3+}$ cluster found in *N*-ethylmaleimide-modified ferredoxin:thioredoxin reductase (NEM-FTR) [11,12]. Both CoM-Hdr and NEM-FTR have the same pattern of positive and negative bands with equivalent bands shifted to lower energy by $\sim$2000 cm^{-1} in CoM-Hdr indicating that each contains a similar type of novel [4Fe–4S]$^{3+}$ cluster.

For NEM-FTR it has been proposed that upon reduction of the active-site disulfide it is cleaved and the one-electron-reduced intermediate is stabilized by attachment of the proximal cysteine to the cluster. The crystal structure of FTR suggests that a cluster Fe is the most likely point of attachment to yield an intermediate involving a [4Fe–4S] cluster with a five-coordinate Fe site [13]. For Hdr several lines of evidence argue in favor of a mechanism involving direct interaction of the heterodisulfide substrate with the active-site [4Fe–4S] cluster rather than the FTR-type mechanism in which cleavage of the substrate disulfide by the [4Fe–4S] cluster is mediated by an active-site disulfide in close proximity to the cluster. First, the marked differences in the redox and electronic excited state properties of the [4Fe–4S]$^{3+}$ clusters in CoB-Hdr and CoM-Hdr argue for direct attachment to the cluster (see above). Second, the [4Fe–4S]$^{3+}$ species in Hdr are readily formed under oxidizing conditions on addition of exogenous thiols such as CoM-SH, CoB-SH, dithiothreitol (DTT) or β-mercaptoethanol [8]. This does not occur in FTR since the active-site disulfide that is present in oxidized samples can only be cleaved under reducing conditions using the physiological electron donor, reduced ferredoxin, or mediator dyes such as reduced viologens [14]. The [4Fe–4S]$^{3+}$ species in FTR is only observed as a stable species on oxidation when one of the active-site cysteine residues has been alkylated, and therefore not available to reform the active-site disulfide on oxidation, leaving the free cysteine available to interact with the cluster. Third, *M. marburgensis* Hdr is not inhibited by cysteine-alkylating reagents at concentrations up to 2 mM [8], whereas cysteine-alkylating reagents are potent inhibitors of FTR as a result of alkylation of the interchange thiol of the active-site disulfide [15]. To obtain further evidence for a direct interaction of CoM-SH with the active-site [4Fe–4S] cluster in Hdr, CoM-^{33}SH was synthesized and was used as substrate for the formation of CoM-Hdr. In addition 2-selanylethane-sulfonate (CoM-SeH) was used as substrate of Hdr.

2. Materials and methods

[^{33}S]S_8 was purchased from Campro Scientific, Berlin, Germany. The analysis of (isotopic) purity of [^{33}S]S_8 was performed by the Kurchatov Institute, Moscow, Russia (isotopic purity: 99.79%; purity: >99.95%).

2.1. Synthesis of coenzyme M analogues (CoM-^{33}SH and CoM-SeH)

The ammonium salt of [2-^{33}S]2-mercaptoethane-sulfonate was prepared in a one-pot procedure from [^{33}S]KSCN (synthesized from [^{33}S]S_8 and KCN) and 2-bromoethane-sulfonate followed by alkaline hydrolysis. CoM-SeH was freshly prepared prior to the enzyme assay from the corresponding diselenide by DTT reduction. The diselenide was synthesized by $NaBH_4$ reduction of 2-selenocyanoethane-sulfonate following a method described by Krief et al. [16]. The details of the optimizations, the spectroscopic characterizations substantiated by independently synthesized reference compounds and the stability tests under assay conditions will be published separately.

2.2. Sample preparation and handling

M. marburgensis was cultured in a 10-l fermenter at 65°C on 80% H_2/20% CO_2/0.1% H_2S as previously described [17]. *M. barkeri* strain Fusaro (DSMZ 804) was cultivated at 37°C on methanol as described in [18]. Cells were harvested under exclusion of oxygen and stored at −80°C. Hdr was purified from the two organisms under strictly anaerobic conditions under an atmosphere of N_2/H_2 (95%/5%) at room temperature using the published protocols [8,19]. All buffers contained 2 mM DTT. Proteins were judged to be >95% pure by SDS–PAGE. The protein concentration was determined using the method of Bradford [20] with bovine serum albumin (Serva) as standard. The method was calibrated by a quantitative amino acid analysis. Prior to spectroscopic measurements of *M. marburgensis* and *M. barkeri* Hdr, DTT was removed by ultrafiltration. Hdr from *M. marburgensis* was in 50 mM Tris–HCl pH 7.6; Hdr from *M. barkeri* was in 50 mM MOPS/KOH pH 7.0 containing 2 mM dodecyl-β-D-maltoside.

For spectroscopic measurements, the enzymes were concentrated to 50 μM by ultrafiltration using a centricon microconcentrator with a 100-kDa cut-off (Millipore). The CoM-Hdr samples were made by oxidizing the protein with 1 mM duroquinone ($E^{\circ\prime} = +86$ mV vs. NHE) followed by addition of 2 mM CoM-SH (Merck) or 2 mM CoM^{33}SH, and incubation at room temperature for 5 min. For treatment of Hdr with CoM-SeH, CoM-Se–Se-CoM (40 mM) was incubated with DTT (20 mM) for 1 h at room temperature and then added to duroquinone-oxidized Hdr to a final CoM-SeH concentration of 2 mM.

2.3. Spectroscopic measurements

EPR spectra at X-band (9 GHz) were obtained with a Bruker EMX spectrometer. All spectra were recorded with a field modulation frequency of 100 kHz. Cooling of the sample was performed with an Oxford Instruments ESR 900 flow cryostat with an ITC4 temperature controller.

Spin quantitations were carried out under non-saturating conditions using 10 mM copper perchlorate as the standard (10 mM $CuSO_4$; 2 mM $NaClO_4$; 10 mM HCl). EPR signals were simulated using home-made programs based on formulas described earlier [21].

3. Results and discussion

Hdr from *M. marburgensis* or *M. barkeri* when oxidized with duroquinone ($E^{\circ\prime} = +86$ mV) is EPR-silent [8]. When CoM-SH is added in the presence of an excess of the oxidant a novel $S = 1/2$ resonance is formed (Fig. 1) which can be detected at temperatures below 50 K, with principal g values $g_{xyz} = 2.013$, 1.991 and 1.938 for Hdr from *M. marburgensis* and $g_{xyz} = 2.012$, 1.993, 1.946 for the *M. barkeri* enzyme. This species has been designated CoM-Hdr [8]. To further address the question if CoM-SH is attached to the cluster in CoM-

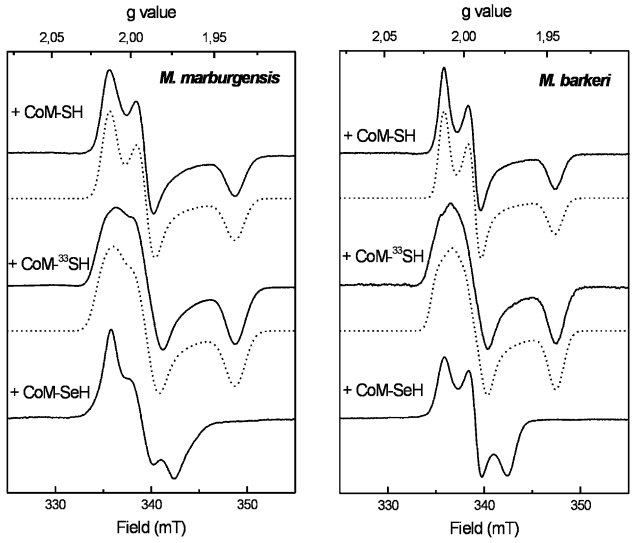

Fig. 1. Comparison of the EPR spectra of oxidized Hdr from *M. marburgensis* (left panel) or *M. barkeri* (right panel) treated with either CoM-SH containing the natural abundance mixture of sulfur, CoM-^{33}SH or CoM-SeH. Solid lines are EPR spectra, dotted lines are the respective simulations. Hdr (50 μM) was oxidized with 1 mM duroquinone and then treated with CoM-SH (2 mM), CoM-S^{33}H (2 mM) or CoM-SeH (2 mM). EPR conditions: temperature 30 K; microwave power, 0.201 mW; microwave frequency 9.457 GHz; modulation amplitude, 0.6 mT. Simulation values: *M. marburgensis*: g_{xyz} = 2.0132, 1.9905, 1.9380; W_{xyz} = 1.50, 1.50, 1.90 mT; (^{33}S, N = 3/2) A_{xyz} = 0.70, 0.58, 0.30 mT. *M. barkeri*: g_{xyz} = 2.0120, 1.9930, 1.9455; W_{xyz} = 1.05, 1.05, 1.50 mT; (^{33}S, N = 3/2) A_{xyz} = 0.70, 0.58, 0.18 mT.

Hdr, CoM^{33}SH, more than 99% enriched in ^{33}S in its thiol group, was synthesized and used as substrate of the enzyme. Fig. 1 shows a comparison of the EPR spectra of *M. marburgensis* Hdr (left panel) and *M. barkeri* Hdr (right panel) incubated with CoM-SH or CoM^{33}SH under oxidizing conditions. With both enzymes an anisotropic broadening of the EPR spectrum was obtained that resulted from an anisotropic hyperfine coupling between the ^{33}S nucleus, which has a nuclear spin 3/2, and the paramagnetic center. From EPR simulations (Fig. 1, dotted lines), hyperfine coupling constants of 0.70, 0.58, 0.18 mT and 0.70, 0.58 and 0.30 mT were estimated for CoM-^{33}SH-treated *M. barkeri* and *M. marburgensis* Hdr, respectively.

In addition to the experiments with CoM-^{33}SH the binding of the substrate analogue seleno-coenzyme M to Hdr was investigated. With Hdr from both organisms, *M. barkeri* and *M. marburgensis*, addition of CoM-SeH to the oxidized enzyme resulted in a new EPR spectrum with principal g values of 2.012(0), 1.993(1) and 1.973(6) (*M. marburgensis* Hdr) and g values of 2.011(7), 1.992(8) and 1.973(2) (*M. barkeri* Hdr). These signals could be observed under non-saturating conditions between 5 and 30 K (Fig. 1). At higher temperatures the signal started to broaden and at 70 K it was broadened beyond detection. This behavior is similar to that of the other CoM-SH- or CoB-SH-induced EPR signals.

Hdr catalyzes the two-electron reduction of a disulfide by a one-electron carrier, an Fe–S cluster. Based on our previous work, we have recently proposed two alternative mechanistic schemes for the reversible heterodisulfide/dithiol cleavage reaction catalyzed by Hdr in two one-electron steps [9]. Both mechanisms involve the one-electron reduction of a [4Fe–4S]$^{2+}$ in the resting enzyme to the [4Fe–4S]$^{1+}$ which immediately reacts to cleave the CoM-S–S-CoB substrate via a nucleophilic substitution reaction. The reaction results in the formation of CoB-SH and a [4Fe–4S]$^{3+}$ cluster with CoM-S^{-} either attached to one iron of the cluster forming a five-coordinated Fe site or to a cluster μ_3-S. The finding that CoM-^{33}SH results in a broadening of the EPR signal of CoM-Hdr provides strong evidence for a direct binding of CoM-SH to the cluster. This relatively strong broadening also allows a prediction of the site of cluster attachment of CoM-SH. In [4Fe–4S] clusters the spin density mainly resides on the Fe rather than on the bridging sulfides [22]. Hence, binding of CoM-^{33}SH to a cluster sulfide will probably result in a very small amount of spin density residing on the sulfur of coenzyme M, only resulting in a small hyperfine splitting of the EPR signal. The data obtained are therefore more consistent with an attachment of CoM-SH to an iron site.

With the substrate analogue seleno-coenzyme M a new EPR signal was obtained again indicative for direct binding of the compound to the active-site cluster of Hdr. The shift of the g values is not directly clear but probably indicates that more spin density resides on the selenium atom giving the signal more characteristics of a radical signal. In future experiments the CoM-SeH-induced Hdr signals will be further characterized using selenium K-edge X-ray absorption spectroscopy, giving direct evidence for the existence of a Fe–Se (or S–Se) bond.

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